Anti-CD99 scFv-ELP nanoworms for the treatment of acute myeloid leukemia

Vijaya Pooja Vaikari[1,#] and Mincheol Park[2,#], Lena Keossayan[1], J. Andrew MacKay[2,3,4], Houda Alachkar[1,5 *]

Affiliations

[1]Department of Clinical Pharmacy, School of Pharmacy, University of Southern California, Los Angeles, CA 90089, United States

[2]Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90089, United States

[3]Department of Ophthalmology, USC Roski Eye Institute, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, United States
[4]Department of Biomedical Engineering, Viterbi School of Engineering, University of Southern California, Los Angeles, CA 90089, United States
[5]USC Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA 90089, United States

 # both authors contributed equally to the manuscript

***Corresponding Author information**:

Houda Alachkar, PharmD, Ph. D

Assistant Professor

University of Southern California, School of Pharmacy

1985 Zonal Avenue John Stauffer Pharmaceutical Sciences Center Room 608

Los Angeles CA 90089

Telephone: 323-442-2696, Email: alachkar@usc.edu

Key Words: CD99, AML, ELP, therapeutic target

Supplementary materials:

The supplemental materials include supplemental methods, table, and figures.

Supplementary methods

Cloning of α-CD99-A192

To generate an elastin-like polypeptide (ELP) targeting CD99, the α-CD99 scFv gene was fused to the amino terminus of an ELP called A192, in the pET-25b(+) vector, encoding α-CD99-A192. The α-CD99 scFv DNA sequence is:

ATGGCTGAAGTACAGTTAGTGGAATCAGGAGGTGGTTTAGTACGCCCAGGTGGTTCTTTAC
GCCTTAGCTGTGCAGCTTCTGGCTTCACATTTAGTTCCTACGCAATGAGTTGGGTCCGTCA
AGCGCCGGGCAAAGGATTAGAGTGGGTGAGCGCAATTAGCGGTTCAGGGGGTTCGACCT
ACTACGCAGACAGTGTCAAGGGTCGCTTTACAATTTCTCGCGATAATTCTAAGAATACCTTG
TATTTGCAAATGAACTCGCTTCGTGCGGAAGATACTGCAGTGTACTATTGTGCAAAAAGCC
ACAAACGCTTTGACTACTGGGGACAGGGAACCTTAGTGACGGTTTCACGCGGAGGCGGTG
GATCTGGCGGCGGAGGGTCGGGGGGGAGGTGGCTCATCAGAATTGACACAGGACCCTGCC
GTGTCAGTTGCCCTGGGGCAAACCGTACGCATTACATGCCAGGGTGATTCACTTCGTTCTT
ACTATGCTTCCTGGTACCAGCAAAAGCCAGGCCAAGCCCCCGTGCTGGTTATCTATGGGA
AAAACAACCGCCCGTCGGGCATTCCAGACCGCTTTTCCGGTTCTTCTTCAGGTAATACGGC
AAGTCTGACCATTACTGGAGCACAAGCCGAGGACGAGGCAGACTACTATTGTAATTCGAGT
TTCCCTCGCACCAGTTCCGTAGTGTTTGGGGGCGGAACTAAATTGACAGTGCTTGGA. The α-CD99 scFv sequence (Integrated DNA Technologies, IA, USA), was cloned into an empty pET-25b(+) vector cut using NdeI and BamHI restriction enzymes (New England Biolabs, MA, USA). The sequence of α-CD99 scFv in pET-25b(+) vector was confirmed, and then BseRI and BssHII restriction enzymes were used to digest pET-25b(+)-α-CD99 scFv and pET-25b(+)-A192 to construct pET-25b(+)-α-CD99-A192. After the ligation, a diagnostic DNA digestion was performed using NdeI and BamHI restriction enzymes followed by electrophoresis on a 1% agarose gel in order to confirm whether the ligation was successful. After the diagnostic DNA digestion, the

plasmid was sent for DNA sequencing with the T7 promoter (TAATACGACTCACTATAGGG) and T7 terminator (GCTAGTTATTGCTCAGCGG), which confirmed in-frame insertion into the pET-25b(+).

α-CD99-A192 Purification

To produce the fusion protein, Clearcoli® BL21 (DE) Electrocompetent Cells (60810, Lucigen, WI, USA) were transformed with the α-CD99-A192 plasmid using electroporation. After transformation, colonies were picked and cultured in 60 ml of Terrific Broth with Glycerol (TB) (C8153, CulGeneX, CA, USA) with 100 μg/ml of carbenicillin at 37 °C for 16-18 hours. For 1 L of TB with 100 μg/ml carbenicillin, 10 mL of Clearcoli® BL21 culture was added. A total of 6 L of bacteria was cultured at 37 °C until the optical density (OD) at 600 nm reached between 0.6 and 0.8. Once the measured OD_{600} reached above 0.6, 400 μl of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to each 1 L flask to bring the final concentration to 400 μM IPTG. IPTG induction was allowed to proceed overnight at room temperature. The next day, bacteria were recovered by centrifugation at 4,000 rpm for 15 minutes, and the supernatant was discarded. Each pellet was resuspended with 30 ml of cold PBS, vortexed, and disrupted using a probe-tip Misonix sonicator S-4000 (Misonix, NY, USA). After sonication, 0.5 % of polyethyleneimine (PEI) was added to each cell lysate, and the cell lysate was incubated on ice for 5 minutes. Lysates were then centrifuged at 13,000 rpm for 15 minutes at 4 °C. The supernatant was recovered, and solid NaCl was added to the supernatant to reach a final concentration of 2 M. Each supernatant with 2 M of NaCl was placed in the water bath at 37 °C until phase separation was observed. After the phase separation, the supernatant was centrifuged at 4,000 rpm for 15 minutes at 37 °C, and the supernatant was discarded. The pellet was resolubilized with cold PBS on ice, and the solubilized pellet was centrifuged at 13,000 rpm for 15 minutes at 4 °C. The supernatant was collected, and this process,

hot and cold centrifugation, was repeated three times with a decreasing volume of PBS and NaCl concentration to remove impurity and increase the purity of α-CD99-A192.

α-CD99-A192 protein refolding

As α-CD99-A192 has four cysteine, the fusion protein was refolded as previously reported to maximize biological activity. The same volume of 8 M urea buffer with 10 mM β-mercaptoethanol (BME) as the volume of α-CD99-A192 in PBS was added. After mixing the 8 M urea buffer with the fusion protein, the mixture was dialyzed to 3 M urea buffer with 2 mM glutathione (GSH) and 0.4 mM oxidized glutathione (GSSH) for 24 hours at 4 °C. Protein was dialyzed to 1 M urea buffer with 2 mM GSH and 0.4 mM GSSH, 0.5 M urea buffer, 0 M urea buffer, and then 2 times of PBS for 24 hours for each step at 4 °C.

α-CD99-A192 competitive binding study

To confirm the specificity of α-CD99-A192 to its target, A192 and α-CD99-A192 were labeled with N-Hydroxysuccinimide-rhodamine (NHS-rhodamine) (Thermo Fisher, MA, USA). The proteins were mixed with 5-fold molar excess of NHS-rhodamine for 2 hours at the room temperature. After the labeling, the mixtures were run through Zeba™ Spin Desalting Columns (Thermo Fisher, MA, USA) using centrifugation at ~1,000×g for 5 minutes to remove unbound NHS-rhodamine. Absorbance was measured to quantify the rhodamine concentration for labeled ELPs using the following equation:

$$C_{ELP} = \frac{A_{555}}{\varepsilon_{Rhodamine} \times l} \quad (1)$$

Where C_{ELP} is the concentration of rhodamine-labeled proteins, A_{555} is absorbance at 555 nm, $\varepsilon_{Rhodamine}$ is molecular extinction coefficient of NHS-rhodamine, which is 80,000 L mol^{-1} cm^{-1}.

Based on these measurements, the degree of labeling was ~1.2, ~1.8 rhodamine per A192, α-CD99-A192 respectively.

Binding of A192 or α-CD99-A192 to cell-surface CD99 was assessed by flow cytometry. 0.5×10^5 cells MOLM-13, MV4-11, U937 or CD99 Null 293T cells were incubated with rhodamine-labeled A192 or α-CD99-A192 (1 and 10 μM) for 30 minutes on ice. Unbound antibody was washed away and bound rhodamine-labeled A192 and α-CD99-A192 were analyzed by measuring the shift in the mean fluorescence intensity (MFI). Data were analyzed by normalizing MFI to untreated cells.

For the competitive binding assays, 0.5×10^5 MOLM-13 cells were preincubated with IgG, mAbCD99 at 2.5 μg/mL and 5 μg/mL for 30 mins on ice. Cells were then washed and treated with rhodamine-labeled α-CD99-A192 (10 μM) for 30 mins on ice. Unbound α-CD99-A192 was washed away and binding of α-CD99-A192 was analyzed by measuring the shift in the (MFI by flow cytometry. Cells were also transferred to glass-bottom MatTek plates (P35G-0.170-14-C, MatTek Corporation, MA, USA) for live cell imaging using ZEISS LSM 880 Confocal Laser Scanning Microscope (Zeiss, Germany) using a 63x objective. Images were quantified with ImageJ. Experiments were performed in triplicate.

Cell lines and primary blasts

All the AML cell lines were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) and 100U/mL penicillin. Peripheral blood mononuclear cells (PBMCs) from patients with AML and healthy donor were isolated by density gradient centrifugation using Ficoll-Paque and cultured in in RPMI supplemented with 20%FBS and cytokine cocktails CC100.

Viability assays

Cell viability was analyzed by incubating 0.5×10^5 cells with either A192 alone or α-CD99-A192 at 1, 10, 25 and 50 μM. Cells were treated for 30 minutes on ice and seeded in a 12 well plate at 5×10^5/mL. Live cells were counted using trypan blue (Life Technologies, Carlsbad, USA) at 72 hours, and normalizing the number of live cells in treated samples to untreated cells. Experiments were performed in triplicates. To determine the IC_{50}, cells were seeded in triplicates in a 96 well plate at 5×10^5 cells/mL and treated with α-CD99-A192 (0.01, 0.1, 0.25, 0.5, 1, 10, 25, 50, and 100 μM). Viability was measure at 72 hours with alamar blue (Invitrogen, Carlsbad, USA) and fluorescence was measured on synergy H1 microplate reader. The change in viability was calculated by normalizing the fluorescence of cells treated with A192 and α-CD99-A192 to that of untreated cells. IC_{50} was calculated based on non-linear regression.

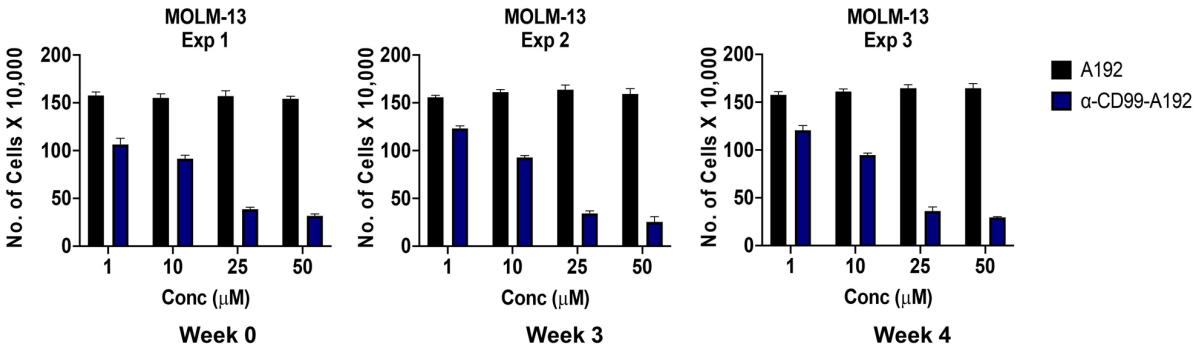

Figure S1. α-CD99-A192 has anti-leukemic activity in AML cells and displays stability when stored at 4 °C. Triplicate trypan blue viability assay performed in MOLM-13 cells treated with α-CD99-A192 or an A192 control for 72 hours using α-CD99-A192 stored at 4 °C.

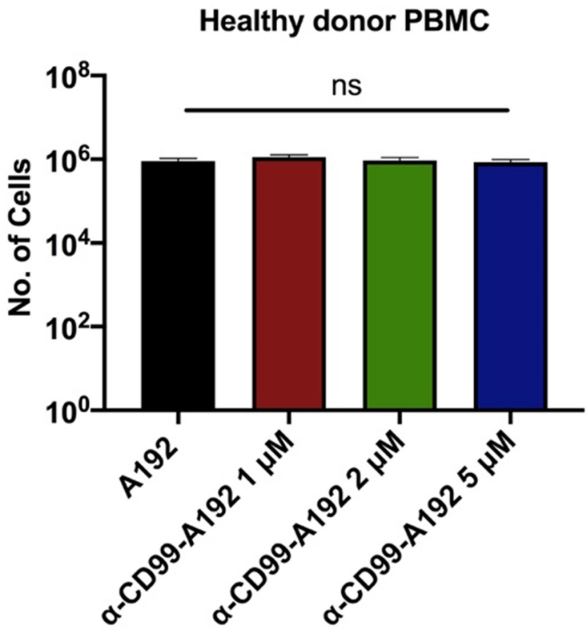

Figure S2. Effect of α-CD99-A192 on healthy donor PBMC. Healthy donor PBMCs were treated with α-CD99-A192 at 1, 2 and 5 μM and live cells were counted using trypan blue at 72 hours.

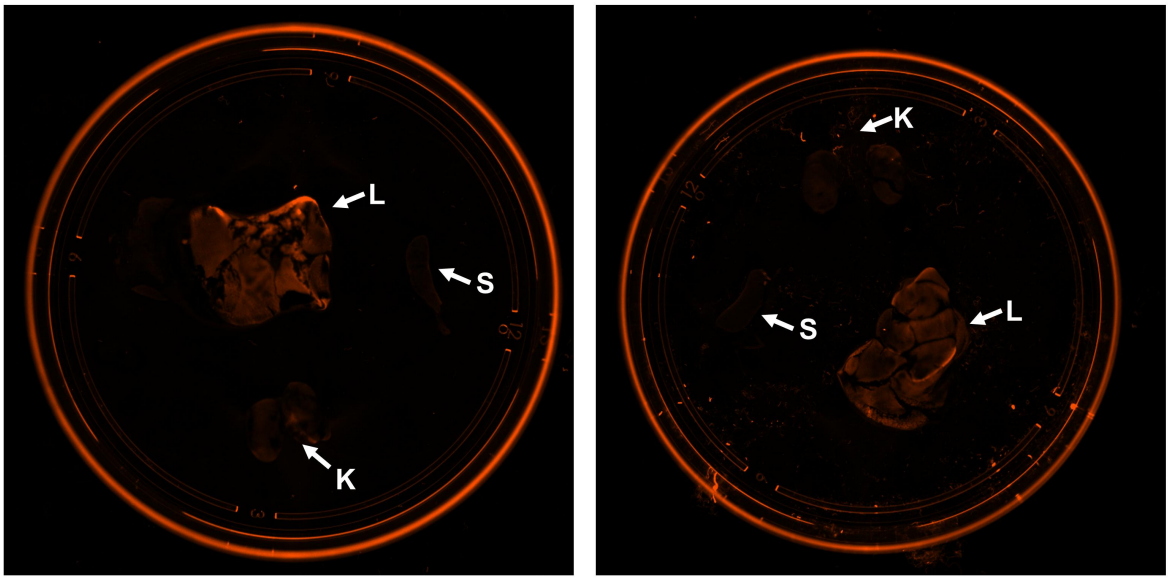

Figure S3. Accumulation of α-CD99-A192 in the liver, kidneys, and spleen. Bioluminescence images of organs from two mice were obtained 96 hours post injection and quantified based on fluorescent intensity obtained from images. L: Liver S: Spleen K: Kidney

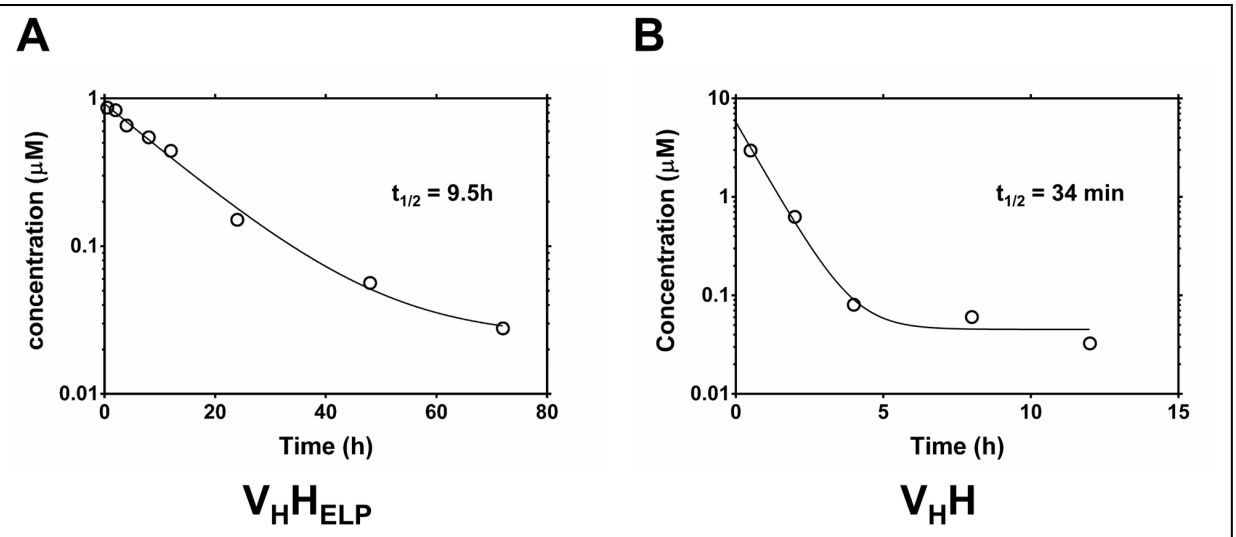

Figure S4. Pharmacokinetic analysis for a published fusion: An ELP fused to a single-domain antibody significantly increases the half-life by decreasing the clearance. To support the influence of an ELP fusion to α-CD99 scFv may increases the half-life, PK data of an ELP fusion protein were extracted from a research paper (Conrad et al, 2011, *Plant Biotechnology J*) and re-analyzed to obtain comparable PK parameters comparable to those of α-CD99-A192. For the reported data, the fusion protein and a single-domain antibody fit a one-compartment model that reached a plateau at a limit of detection. The fusion protein extended the half-life of the single-domain antibody by substantially decreasing the clearance (**Table S1**), similarly to α-CD99-A192.

Table S1. ELP attachment to a single-domain antibody reduces clearance and extends the mean residence time[1]

Parameter (Unit)	V_HH	V_HH_{ELP}
Dose (nmol)	5.95	1.75
AUC (μM*h)[a]	3.89	14.6
AUMC (μM*h^2)[a]	7.33	258
MRT (h)[a]	1.88	17.7
CL (ml/h)[a]	1.53	0.12
V_{ss} (ml)[a]	2.88	2.13
C_0 (μM)[b]	5.72	0.90
Plateau (μM)[b]	0.045	0.024
$t_{1/2}$ (h)[b]	0.57	9.5
$k_{elimination}$ (h^{-1})[b]	1.21	0.07
AUC (μmol*h)[b]	4.73	12.3
CL (ml/h)[b]	1.26	0.14
V_d (ml)[b]	1.04	1.95

[a]determined using non-compartmental analysis
[b]determined by fitting to a single exponential decay to a plateau (Supplemental Fig. S3) and fit to a one-compartment model of an intravenous bolus[2]

References

1. Conrad U, Plagmann I, Malchow S, Sack M, Floss DM, Kruglov AA, et al. ELPylated anti-human TNF therapeutic single-domain antibodies for prevention of lethal septic shock. *Plant Biotechnol J*. 2011;**9**:22-31.
2. Guo H, MacKay JA. A pharmacokinetics primer for preclinical nanomedicine research. Nanoparticles for Biomedical Applications: Elsevier; 2020. p. 109-28.